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(71) Applicant (*for all designated States except US*): UNIVERSITY OF HAWAII [US/US]; 2800 Woodlawn Drive, Honolulu, HI 96822 (US).

(71)(72) Applicants and Inventors: STILES, John, I. [–/US]; 46-372 KumooLoop, Kaneohe, HI 96744 (US). MOISYADI, Istefo [–/US]; Apartment 3213, 1717 Mott-Smith Drive, Honolulu, HI 96822 (US). NEUPANE, Kabi, Raj [–/US]; 2724 Kolo Place, Honolulu, HI 96826 (US).

(74) Agents: GRIFFITH, Calvin, P. et al.; Jones, Day, Reavis & Pogue, North Point, 901 Lakeside Avenue, Cleveland, OH 44114 (US).

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(54) Title: PURIFIED PROTEINS, RECOMBINANT DNA SEQUENCES AND PROCESSES FOR CONTROLLING THE RIPENING OF COFFEE PLANTS

(57) Abstract

Purified proteins, DNA sequences that code on expression therefore and recombinant DNA molecules, including hosts transformed therewith for transforming coffee plants to suppress the expression of enzymes necessary for ethylene synthesis. The DNA sequences and recombinant DNA molecules are characterized in that they code on expression for the enzymes ACC synthase or ACC oxidase that are elements of the pathway for ethylene biosynthesis in coffee plants. Coffee plants are tansformed with vectors containing ACC synthase and/or with ACC oxidate DNA sequences that code on expression for the respective mRNA that is antisense to the mRNA for ACC synthase and/or ACC oxidase. The resulting antisense mRNA binds to XMI mRNA, thereby inactivating the mRNA encoding one or more enzymes in the pathway for ethylene synthesis. The described DNA sequences can also be used to block synthesis of ACC synthase or ACC oxidate using co-suppression. The result in either event is that the transformed plants are incapable of synthesizing ethylene, though other aspects of their metabolism is not affected.

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PURIFIED PROTEINS, RECOMBINANT DNA SEQUENCES AND PROCESSES FOR CONTROLLING THE RIPENING OF COFFEE PLANTS

FIELD OF THE INVENTION

This application relates to purified proteins, recombinant DNA sequences, hosts transformed therewith and processes for controlling the ripening of coffee plants. More particularly, this application relates to purified proteins, and recombinant DNA sequences that can be used to suppress the expression of coffee fruit-specific 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase genes. This application further relates to coffee plants transformed with such sequences, thereby rendered incapable of synthesizing ethylene necessary for ripening. Application of exogenous ethylene to plants transformed in accordance with this invention makes it possible to synchronize and control fruit ripening in coffee plants.

BACKGROUND OF THE INVENTION

Coffee is prepared from the roasted beans of the plants of the genus *Coffea*, generally from the species *C. arabica*. Beans are the seeds of the coffee plant and are obtained by processing the fruit, most ideally mature fruit which commands the

best price due to its superior quality. In the past, high quality "gourmet" coffee was hand picked. This is necessary because the fruits of a coffee tree do not ripen uniformly and thus there are both mature and immature fruit on the same tree. In the past, this was not a serious problem as most coffee is grown in areas of the world where labor is plentiful and not expensive. However, more recently lack of abundant and inexpensive labor has become a major contributor to decreased productivity in coffee production. To increase productivity some regions of the world, such as the largest coffee producing country, Brazil, have resorted to strip harvesting where workers rapidly remove all fruit from a branch whether ripe or unripe. This increases the speed of harvesting but decreases the yield of the highest quality beans as much of the fruit is immature (green).

Furthermore, the lack of uniform ripening has seriously limited the effectiveness of mechanical harvesting. The force required to remove mature fruit (cherry) from the tree is similar to the force required to remove green fruit. Thus, mechanical harvesters do not distinguish well between green and cherry and a large amount of immature fruit is harvested along with mature fruit. This greatly

decreases the yield of mature fruit and limits productivity. If coffee fruit ripening could be controlled so that all fruit ripened at one time, both the strip method of hand harvesting and mechanical harvesting would be much more efficient and a higher percentage of the harvested fruit would be in the higher quality grades. This would increase profitability of coffee production.

As is the case with many other fruit [Yang and Hoffman, Ann. Rev. Plant Physiol. 35:155 (1984)], plant-produced ethylene plays an important role in the final stages of fruit ripening in coffee. Once coffee fruit reach a certain stage of maturity they can be induced to ripen by the exogenous application of ethylene [Crisosto, C.H., P.C. Tausend, M.A. Nagao, L.H. Fuchigami and T.H.H. Chen, J. Haw. Pac. Agri. 3:13-17 (1991)]. This demonstrates the importance of ethylene for the final stages of fruit ripening in coffee.

Ethylene is synthesized in a two-step reaction from S-adenosylmethionine (SAM). The first step is the synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) from SAM by ACC synthase. In most plants this is the rate limiting step. The final step is the conversion of ACC to ethylene which is catalyzed by ACC oxidase (Yang and Hoffman, *supra*).

Inhibition of ethylene biosynthesis by chemical (*e.g.*, silver ions or carbon dioxide) or biotechnological means [Oeller *et al.*, Science 254:437 (1991)] inhibits the final stages of ripening. This inhibition is reversible by the application of ethylene.

Accordingly, a strategy for controllmg the ripening of coffee plants is to prevent synthesis of specific enzymes in the pathway for ethylene biosynthesis. In one embodiment this invention relates to genetic alteration of coffee plants to eliminate synthesis of ACC synthase; in another, ACC oxidase synthesis is suppressed. In the presently preferred embodiments, synthesis of one or both of these enzymes is suppressed by transforming coffee plants with a DNA sequence that codes on transcription for a messenger RNA (mRNA) that is antisense to the mRNA that codes on expression for the enzyme whose synthesis is to be suppressed. See Oeller *et al.*, Science 254:437 (1991), who reported controlling ripening of tomatoes using a similar strategy.

Recombinant DNA technology has been used to isolate a number of ACC synthase and ACC oxidase genes. However, the genes for ACC synthase and ACC

oxidase in coffee have not been identified or sequenced to date.

SUMMARY OF INVENTION

Purified proteins, DNA sequences that code on expression therefore and recombinant DNA molecules, including hosts transformed therewith, for transforming coffee plants to suppress the expression of enzymes necessary for ethylene synthesis. The DNA sequences and recombinant DNA molecules are characterized in that they code on expression for the enzymes ACC synthase or ACC oxidase that are elements of the pathway for ethylene biosynthesis in coffee plants.

Coffee plants are transformed with vectors containing ACC synthase and/or with ACC oxidase DNA sequences inserted so that the transforming sequences code on expression for the respective RNA that is antisense to the mRNA for ACC synthase and/or ACC oxidase. The resulting antisense RNA binds to mRNA(s), thereby inactivating the mRNA encoding one or more enzymes in the pathway for ethylene synthesis. The described DNA sequences can also be used to block synthesis of ACC synthase or ACC oxidase using co-suppression. The result in either event is that the transformed plants are

incapable of synthesizing ethylene, though other aspects of their metabolism is not affected.

Ripening in the transformed plants can be regulated by exogenous ethylene. By application of ethylene to the entire plant, the entire plant will ripen at once, making mechanical harvesting of coffee more productive.

SUMMARY OF THE DRAWINGS

FIGURE 1 is the complete sequence of the cDNA encoding coffee fruit expressed ACC synthase.

FIGURE 2 is the amino acid sequence of the coffee fruit ACC synthase deduced from the cDNA sequence shown in FIGURE 1.

FIGURE 3 is the sequence of the cDNA encoding coffee fruit expressed ACC oxidase.

FIGURE 4 is the amino acid sequence of the coffee fruit ACC oxidase deduced from the cDNA sequence shown in FIGURE 3.

DETAILED DESCRIPTION OF THE INVENTION

In order that the invention herein described may be more fully understood, the following detailed description is set forth. In the description the following terms are employed:

Nucleotide -- A monomeric unit of DNA or RNA consisting of a sugar moiety (pentose), a phosphate, and a nitrogenous heterocyclic base. The base is

linked to the sugar moiety via the glycosidic carbon (1' carbon of the pentose) and that combination of base and sugar is called a nucleoside. The base characterizes the nucleotide. The four DNA bases are adenine ("A"), guanine ("G"), cytosine ("C"), and thymine ("T"). The four RNA bases are A, G, C, and uracil ("U").

DNA Sequence -- A linear array of nucleotides connected one to the other by phosphodiester bonds between the 3' and 5' carbons of adjacent pentoses.

Codon -- A DNA sequence of three nucleotides (a triplet) which encodes through mRNA an amino acid, a translation start signal or a translation termination signal. For example, the nucleotide triplets TTA, TTG, CTT, CTC, CTA and CTG encode for the amino acid leucine ("Leu"), TAG, TAA and TGA are translation stop signals and ATG is a translation start signal, which also encodes the amino acid methionine ("MET").

Polypeptide -- A linear array of amino acids connected one to the other by peptide bonds between the amino and carboxy groups of adjacent amino acids.

Genome -- The entire DNA of a cell or a virus. It includes inter alia the structural gene coding for the polypeptides of the substance, as well as

promoter, transcription and translation initiation and termination sites.

Gene -- A DNA sequence which encodes through its template or messenger RNA ("mRNA") a sequence of amino acids characteristic of a specific polypeptide.

Transcription -- The process of producing mRNA from a gene or DNA sequence.

Translation -- The process of producing a polypeptide from mRNA.

Expression -- The process undergone by a gene or DNA sequence to produce a polypeptide. It is a combination of transcription and translation.

Plasmid -- A nonchromosomal double-stranded DNA sequence comprising an intact "replicon" such that the plasmid is replicated in a host cell. When the plasmid is placed within a unicellular organism, the characteristics of that organism may be changed or transformed as a result of the DNA of the plasmid. For example, a plasmid carrying the gene for tetracycline resistance (TETR) transforms a cell previously sensitive to tetracycline into one which is resistant to it. A cell transformed by a plasmid is called a "transformant."

Phage or Bacteriophage -- Bacterial virus many of which consist of DNA sequences encapsidated in a protein envelope or coat ("capsid").

Cloning Vehicle -- A plasmid, phage DNA, cosmid or other DNA sequence which is able to replicate in a host cell, characterized by one or a small number of endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without attendant loss of an essential biological function of the DNA, e.g., replication, production of coat proteins or loss of promoter or binding sites and which contain a marker suitable for use in the identification of transformed cells, e.g., tetracycline resistance or ampicillin resistance. A cloning vehicle is often called a vector.

Cloning -- The process of obtaining a population of organisms or DNA sequences derived from one such organism or sequence by asexual reproduction.

Recombinant DNA Molecule or Hybrid DNA - A molecule consisting of segments of DNA from different genomes which have been joined end-to-end outside of living cells and able to be maintained in living cells.

cDNA - A DNA strand complementary to an mRNA that codes for a particular polypeptide.

The strategy for controlling ethylene biosynthesis in coffee plants according to the present invention relates in the first instance to determination of the genes that code on expression for two enzymes in the ethylene pathway: ACC synthase and ACC oxidase. Transformation of wild type coffee plants with constructs containing either or both genes in an orientation that is antisense to the normal genes is expected to block synthesis of the respective enzymes. Messenger RNA transcribed under direction from the transforming sequence will bind to mRMA transcribed under direction from the normal sequence, thereby inactivating the normal message and precluding enzyme synthesis.

To isolate the DNA sequences that code on expression for ACC synthase and ACC oxidase in coffee, we screened a cDNA library produced from coffee plant tissue with synthetic DNA probes containing nucleotide sequences expected to occur. These expected sequences were based on studies of nucleotide sequences that occur in genes that encode the respective enzymes, other climacteric plants and other plants.

The cDNA corresponding to the gene encoding ACC synthase or ACC oxidase is used to transform embryonic coffee plants. The plasmid pBI-121 is

used as a transforming vector. The sequences corresponding to DNA that codes on expression for ACC synthase or ACC oxidase is inserted into the plasmid in an inverted orientation adjacent to a cauliflower mosaic virus 35S promoter. RNA transcribed therefrom will be complementary to mRNA that encodes the amino acid sequence of the respective enzyme. Complete constructs are amplified in bacterial hosts. The hosts are disrupted and the amplified vector is attached to colloidal gold particles. The gold particles with adherent vectors are inserted into coffee plant tissue by propelling the particles at high speed at the cells as described in U.S. patent 5,107,065. Young plants successfully transformed are identified by antibiotic resistance. The transformed plants do not produce ACC synthase or ACC oxidase, depending on the gene used to transform the plants. Ripening of the transformed plants is initiated by application of exogenous ethylene.

EXAMPLE 1

Isolation of Coffee Fruit-Specific ACC Synthase cDNA

In order to isolate ACC synthase gene sequences involved in the ripening of coffee, a cDNA library was prepared from a mixture of coffee fruit pericarp and mesocarp tissue at different stages of ripeness.

This library was screened using a PCR product synthesized from first-strand cDNA made from the same mRNA used to construct the library and degenerate oligonucleotide primers corresponding to consensus sequences derived from ACC synthase genes from other organisms. This example principally involved the isolation of mRNA, the construction of a cDNA library, and the subsequent steps involved in cloning the appropriate cDNA.

a) Isolation of mRNA

Total RNA was isolated from 66 g of pericarp and mesocarp tissue from several different developmental stages of coffee fruit (*C. arabica* L. cv Guatemalan) using the method of Levi et al., [Hort Science 27(12):1316-1318 (1992)]. Frozen coffee fruit pericarp and mesocarp tissue was powdered by grinding for about 2 minutes in a domestic coffee mill (Salton Model GC-5; Salton Maxam Housewares Group, Mt. Prospect, IL) with a small piece of dry ice. The powdered fruit tissue was added to 200 μL of 200 mM tris[hydroxymethyl]aminomethane hydrochloride (tris-HCl) (pH 8.5), 1.5% sodium dodecyl sulfate (SDS), 300 mM LiCl, 10 mM disodium ethylenediaminetetraacetic acid (Na_2EDTA), 1.5% sodium deoxycholate (w:v), 1.5% Nonidet P-40 (Sigma

Chemical Co.) (v:v), 0.5 mM thiourea, 1 mM aurintricarboxylic acid, 10 mM dithiothreitol (DTT), 75 mM B-mercaptoethanol, 2% polyvinylpyrrolidone (PVP) and 2% polyvinylpoly-pyrrolidone (PVPP) and homogenized using a Polytron tissue homogenizer (Tekmar, Cincinnati, OH). After 2 minutes of homogenization, 200 μL of chloroform was added and homogenization continued for a further 3 minutes. The homogenate was transferred to 250 μL centrifuge bottles (Nalgene) and centrifuged for 15 minutes at 2,500 x *g*. The upper aqueous phase was removed and mixed with 12 μL of 5 M NaCl, equally divided into two centrifuge bottles, and 150 μL of ethanol was added to each bottle. The mixture was stored at -20°C overnight. The RNA was collected by centrifugation at 4,000 x *g* for 15 minutes at 4°C. The RNA was dissolved in 50 μL TE1 (50 mM tris-HCL [pH 8.0], 10 mM Na_2EDTA) and clarified by centrifugation at 12,000 x *g* for 10 minutes at 4°C. The supernatant was transferred to a new centrifuge bottle and 3 μL of 5 M NaCl and 30 μL of isopropanol were added. The contents were mixed and stored at -20°C overnight. The RNA was collected by centrifugation at 14,000 x *g* for 10 minutes. The RNA was washed with 20 μL of 70% ice-cold ethanol and collected by centrifugation as before. After

drying under vacuum for 10 minutes, the RNA was resuspended in 50 μL of TE1 buffer and 10 μL of 12 M LiCl was added. The solution was incubated at 4°C for 48 hours and the RNA was collected by centrifugation at 14,000 x *g* for 10 minutes and resuspended in 30 μL TE1 buffer. After the addition of 15 μL of 5 M potassium acetate, the RNA was incubated overnight at 0°C, recovered by centrifugation at 14,000 x *g* for 10 minutes and suspended in 50 μL TE1 buffer. Three μL of 5 M NaCl and 110 μL of 95% ethanol were added and the RNA was incubated at -20°C overnight. The RNA was recovered by centrifugation at 14,000 x *g* for 10 minutes, washed with 20 μL of 70% ice-cold ethanol, recovered by centrifugation as above, dried under vacuum for 10 minutes and resuspended in 600 μL of TE1 buffer. The RNA was transferred into a microcentrifuge tube and centrifuged at 14,000 rpm for 30 minutes at 4°C after which 300 μL was removed to each of two new microcentrifuge tubes. The original centrifuged tube was rinsed with an additional 300 μL of TE1 buffer. Eighteen μL of 5 M NaCl and 636 μL of 100% ethanol were added to each of the three tubes. After mixing by inverting, the tubes were stored overnight at -20°C. The RNA was collected by centrifugation at 14,000 rpm for 30 minutes and

washed with 1 μL of 70% ice-cold ethanol. After centrifugation and drying as above, the RNA was resuspended in 400 μL sterile H_2O. A total of 1.04 mg total RNA was obtained.

Messenger RNA (polyA+ RNA) was isolated using the PolyATtract® mRNA Isolation System IV (Promega Corporation, Madison, WI). A total of two isolations were done as follows. For each isolation, 0.48 mg total RNA was dissolved in 800 μL of RNase-free water. After heating at 65°C for 10 minutes, 3 μL of 50 pmole/mL biotinylated oligo(dT) and 20.7 μL of 20 X SSC (1 X SSC contains 150 mM NaCl and 15 mM sodium citrate) were added and the mixture was allowed to slowly cool to room temperature over a period of approximately 30 minutes. An aliquot of streptavidin paramagnetic particles (provided in the PolyATtrack® mRNA Isolation System IV) was washed 3 times in 0.5 X SSC and resuspended in 0.1 mL of 0.5 X SSC. The RNA solution containing the biotinylated oligo(dT) was added to the washed streptavidin paramagnetic particles. After a 10 minute incubation at room temperature, the paramagnetic particles containing the trapped mRNA were captured to the side of the tube using a magnet.

The supernatant was removed and the particles were washed four times with 0.3 mL of 0.1 X SSC. The mRNA was removed from the biotinylated oligo(dT) particles by suspending in 200 μL RNase-free water. An additional elution was carried out by adding 150 μL of water sequentially to each of the two tubes. The elution fractions (550 μL) were pooled and centrifuged at 14,000 rpm in a microcentrifuge for 30 minutes at 4°C. The supernatant was divided into two microcentrifuge tubes and, after the addition of 1/10th volume of 3 M NaCl and 600 μL of ethanol, the mRNA was recovered by incubating the tubes at -20°C overnight, followed by centrifugation as above. The mRNA was washed once with 1 mL of ice-cold 70% ethanol, dried and resuspended in 20 μL sterile H_2O. One μL was added to 1 mL of water and a spectrum was obtained from 230 nm through 330 nm in a Shimadzu UV 160U spectrophotometer. Approximately 6 μg of mRNA was recovered from 1.04 mg of total RNA.

b) Construction of a cDNA Library

First and second strand cDNA was synthesized using the ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA). Six micrograms of mRNA in 20 μL of water were incubated at 65°C for 5 minutes. Two microliters of 100 mM methyl mercury were added and incubation was continued at room temperature for 10

minutes. Four microliters of 700 mM β-mercaptoethanol were added and the incubation was continued for an additional 5 minutes. To the denatured mRNA, 5 μL of 10 X first strand buffer (provided in the kit), 5 μL of 100 mM DTT, 3 μL nucleotide mixture (10 mM each dATP, dGTP, dTTP and 5-methyl-dCTP), 2 μL of 1.4 μg/μL linker-primer:

5'-GAGAGAGAGAGAGAGAGAACTAGTCTCGAGTTTTTTTTTTTTTTTT-3'

(SEQ. ID NO. 1)

1 μL RNase block and 5 μL of water were added. The reaction was incubated at room temperature for 10 minutes to anneal the primer to the mRNA and then 3 μL of 20 U/μL M-MuLV reverse transcriptase were added. Five microliters of this reaction mixture were removed to a tube containing 0.5 μL (0.625 pmoles) of 800 Ci/mmole [α^{-32}P]dATP. Both reactions were incubated at 37°C for 1 hour. The radioactively labeled reaction was frozen at -20° C for later gel analysis. To the 45 μL main reaction, 40 ML of second strand buffer, 15 μL of 100 mM DTT, 6 μL of nucleotide mixture (10 mM dATP, dGTP, dTTP and 26 mM dCTP), 268.3 μL water and 2 μL (2.5 pmoles) of 800 Ci/mmol [α^{-32}P]dATP were added. After mixing, 4.5 μL of 1 U/μL RNase H and 19.2 μL of 5.2 U/μL *E. coli* DNA polymerase I were added and the reaction was incubated at 16° C for 2.5 hours. The

reaction was extracted with 400 μL of phenol:chloroform (1:1). The phases were separated by centrifugation in a microcentrifuge for 5 min and the aqueous phase removed and re-extracted with chloroform. The aqueous phase was recovered by centrifugation as before.

The double-stranded cDNA was precipitated by the addition of 33.3 μL of 3M sodium acetate (pH 5.2) and 867 μL of 100% ethanol and incubation overnight at -20°C. The cDNA was recovered by centrifugation at 14,000 X *g* in a microcentrifuge at 4°C for 60 minutes. The cDNA was washed with 1 mL of 80% ethanol, recovered by centrifugation at room temperature in a microcentrifuge at 14,000 X *g*, dried under vacuum and dissolved in 45 μL of water. Three microliters of the resuspended double-stranded cDNA was removed and stored at -20°C for later analysis by gel electrophoresis.

To the remaining 42 ML of the double-stranded cDNA, 5 μL of 10 X Klenow buffer (buffer #3; supplied by Stratagene), 2.5 μL of 2.5 mM nucleotides (dCTP, dGTP, dATP and dTTP), and 0.5 μL of 5 U/μL *E. coli* DNA polymerase I Klenow fragment were added. After 30 minutes at 37°C, 50 μL of water were added and the reaction was extracted with an equal volume of phenol:chloroform (1:1) and then

chloroform as described above. After the addition of 7 μL of 3M sodium acetate (pH 5.2) and 226 μL of 100% ethanol, the blunt-ended double-stranded cDNA was incubated on ice for 30 minutes and recovered by centrifuging at 14,000 rpm at 4°C for 60 minutes in a microcentrifuge. The cDNA was washed with 300 μL of 70% ethanol, centrifuged and dried as before. Seven microliters of 0.4 μg/μL *Eco*RI linkers were added to the dried cDNA. The structure of the *Eco*RI linkers are:

5'-AATTCGGCACGAG-3' (SEQ. ID NO. 2)

3'-GCCGTGCTC-5'

After vortexing to resuspend the cDNA, 1 μL of 10 X ligation buffer, 1 μL 10 mM ATP and 1 μL of 4 Weiss U/μL T4 DNA ligase were added and the reaction was incubated over night at 8°C. The ligase was inactivated by heating at 70°C for 30 minutes. The 5' ends of the *Eco*RI linkers, that are now attached to the cDNA, were phosphorylated using polynucleotide kinase. One microliter of 10 X buffer #3 of the ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA), 2 μL of 10 mM ATP, 6 μL of water and 1 μL of 10 U/μL T4 polynucleotide kinase were added to the ligation reaction. After 30 minutes at 37°C the kinase reaction was stopped by heating the reaction at 70°C for 30 minutes. *Xho*I "sticky ends"

were generated at the end of the cDNA corresponding to the 3' end of the mRNA by digestion of the *Xho*I site in the linker-primer. Twenty-eight μL of *Xho*I buffer and 3 μL of 40 U/μL *Xho*I were added to the cDNA and the reaction was incubated at 37°C for 1.5 hours.

The cDNA, with *Eco*RI sticky ends at the 5' end and *Xho*I sticky ends at the 3' end (relative to the original mRNA), was size fractionated by passage through a Sephacryl S-400 spin column prepared as follows. Five μL of 10 X STE [100 mM Tris (pH 7.0), 5 mM EDTA and 100 mM NaCl] were added to the cDNA and the cDNA was applied to the top of a 1 mL syringe containing Sephacryl S-400 (Pharmacia Biotech, Piscataway, NJ). A 500 μL microcentrifuge tube was placed on the bottom of the syringe and the column was placed in a centrifuge tube and centrifuged at about 400 X *g* for 2 minutes. Sixty μL of 1 X STE were added to the top of the syringe, a new microcentrifuge tube was placed on the bottom of the column and the column was again centrifuged as before. This process was repeated until six fractions had been collected. About 10% of each fraction was electrophoresed on a 1% agarose gel to determine the size distribution of the cDNA in each fraction. The remainder of each fraction was

extracted with an equal volume of phenol:chloroform and then chloroform as described above and precipitated by the addition of 2 volumes of 100% ethanol. After overnight incubation at -20°C the cDNA was recovered by centrifugation in a microcentrifuge at 14,000 rpm for 60 minutes at 4°C. Each cDNA fraction was washed with 200 NL of 80% ethanol and dried as described above. cDNA fraction 1 was resuspended in 3 μL of sterile water, and cDNA fraction 2 was resuspended in 10.5 μL of sterile water. One-half μL of each of the two fractions was used to determine the quantity of DNA using the ethidium bromide plate detection method. Fractions 1 and 2, containing the largest cDNA molecules, were combined. The 12.5 mL combined fractions contained approximately 100 ng of cDNA. This fraction was reduced to 2.5 μL in a Speed-Vac and stored on ice. cDNA fraction 3 was resuspended in 10.5 μL of sterile water, and saved at -20°C for later use.

One-hundred ng of cDNA from fraction 1 and 2 were ligated into 1 μg of Uni-ZAP™ (Stratagene, La Jolla, CA), a lambda ZAP vector that had been digested with *Eco*RI and *Xho*I. Fraction 1 and 2 cDNA (2.5 μL) were added to 0.5 μL of 10 X ligation buffer, 0.5 μL 10 mM ATP, 1 μL of 1 μg/μL Uni-Zap XR vector and 0.5 μL of 4 Weiss U/μL T4 DNA ligase.

The reaction was incubated at 8°C for about 44 hours. A 1 μL aliquot of the ligation reaction was added to one aliquot of the 'Freeze-Thaw' extract from the Gigapack II Gold bacteriophage λ packaging kit (Stratagene, La Jolla, CA). Fifteen microliters of Sonic extract were added and the contents were gently mixed. The packaging was carried out at room temperature. After 2 hours, 500 μL of SM buffer and 20 μL of chloroform were added to each packaging reaction and the debris was removed by a short centrifugation in a microcentrifuge. The packaged phages were moved to a new microcentrifuge tube. Ten μL of chloroform were added and the packages phages were stored at 4°C until used. A titer of this primary library indicated the presence of 0.7 X 10^6 recombinant plaques.

c) Amplification of primary library.

Six-hundred μL of *E. coli* XL1-Blue MRF' (Stratagene, La Jolla, CA), grown to a density of 0.5 at $O.D._{600}$, and 32.5 μL of primary library stock were added to each of 16 tubes. After incubation at 37°C for 15 min, 6.0 mL of 48°C top agar (5 g/L NaCl, 2 g/L $MgSO_4.7H_2O$, 5 g/L yeast extract, 10 g/L NZ amine [pH 7.5], and 0.7% agarose) were added to each tube and the contents were plated on 150 X 15 mm NZY plates (5 g/L NaCl, 2 g/L $MgSO_4.7H_2O$, 5 g/L

yeast extract, 10 g/L NZ amine [pH 7.5], and 15 g/L Difco agar). The plates were incubated overnight at 37°C and then overlayed with 10 mL of SM buffer and incubated for a further 8 hours at 4°C with gentle shaking. The SM buffer was collected with a sterile pipette and stored in a sterile 250 mL centrifuge bottle. Each plate was rinsed with an additional 10 mL of SM buffer which were collected and added to the previous SM buffer. Chloroform, to a final concentration of 5%, was added and the phage solution was incubated at room temperature for 15 minutes and then centrifuged at 2,000 X *g* for 10 minutes to remove cell debris. The supernatant was recovered to a sterile polypropylene bottle and chloroform was added to a final concentration of 0.3%. The amplified library was stored at 4°C.

d) Plating of amplified library for screening for specific genes.

The amplified library was titered as described above. Approximately 50,000 recombinant plaques were added to 600 µL of *E. coli* XL1-Blue MRF' that were grown as described above. After 15 min at 37°C, 6.5 mL of 48°C top agar were added and the cells were plated on 150 X 15 mm NZY plates. Four plates containing a total of 200,000 recombinant plaques were prepared and incubated at 37°C overnight. The plates were then chilled for 4 hours

at 4°C, then used for preparing plaque lifts as described below.

e) Identification and Construction of Oligonucleotides Homologous to Coffee ACC Synthase Genes

In previous studies, described in United States patent application serial number 08/485,107 the specification of which is incorporated herein by reference, we identified base sequences common to ACC synthase occurring in a variety of plants, referred to herein as consensus sequences. Based on these studies, we developed a set of three (3) fully degenerate primers for PCR amplification of regions of coffee first strand cDNA corresponding to consensus sequences. The sequence of the primers used is:

ACS167: 5'-GCCAAGCTTCCRTGRTARTCYTGRAA-3'

(SEQ. ID NO. 3)

ACS289: 5'-TTYCARGAYTAYCAYGGHYT-3'

(SEQ. ID NO. 4)

ACS885: 5'-CCHGGDARNCCYAWRTCTTT-3'

(SEQ. ID NO. 5)

f) Reverse Transcriptase reaction to obtain first-strand coffee cDNA.

The reverse transcriptase reaction to obtain first-strand cDNA was performed in a final volume of 20 μL using the GeneAmp RNA PCR Core Kit (Perkin

Elmer, Foster City, CA). First, 0.9 μg of coffee fruit mRNA in 3 μL water was mixed with 1 μL of 50 μM random hexamer and 6 μL of sterile water in a microcentrifuge tube and incubated at 65°C for 5 minutes. The mixture was left at room temperature for 2 minutes and the liquid was recovered to the bottom of the tube by a brief centrifugation. To this mixture 2 μL PCR buffer II (from the above mentioned kit), 4 μL 25 mM $MgCl_2$, 2 μL 10 mM dNTP's, 1 μL RNAsin (20 u/μL), and 1 μL reverse transcriptase (50 u/μL) were added. The reaction was incubated at 42°C for 1 hour after which the reverse transcriptase was heat inactivated in a 95°C water bath for 5 minutes.

g) Polymerase chain reaction to amplify coffee ACC-synthase gene.

A polymerase chain reaction (PCR) (Saiki et al., 1988) was performed using the GeneAmp Kit described above in a 50 μL reaction containing 10 μL first-strand cDNA mix, 4 μL PCR buffer II, 1 μL 25 mM $MgCl_2$, 2.5 μL of 20 μM AC5167 primer (SEQ. ID NO. 3), 2.5 μL 20 μM AC5885 primer (SEQ. ID. NO. 5), 29.5 μL sterile H_2O, and 0.5 μL Tag DNA polymerase (5 u/μL). PCR conditions were 35 cycles of 94°C for 1 minute, 44°C for 1 minute, and 72°C for 2 minutes. The product of the PCR reaction was analyzed by agarose gel electrophoresis using 1.5% SeaPlaque

agarose (FMC BioProducts, Rockland, ME) and *Hae* III-digested ϕX174 DNA (Promega Corporation, Madison, WI) as size markers. A single PCR product of approximately 650 bp was obtained.

h) Amplification of PCR product with different primers.

The 650 bp fragment obtained above was excised from the gel and placed in a 1.5 mL microcentrifuge tube. After the addition of 200 μL of sterile water, the 650 bp fragment was heated to 90°C for 5 minutes, cooled to room temperature and centrifuged at 14,000 rpm for 5 minutes in a microcentrifuge. The supernatant containing the amplified DNA was removed and placed in a new sterile 1.5 mL microcentrifuge tube. A 25 μL PCR reaction was carried out using 0.4 μL of the previously amplified DNA as template, 2.5 μL 10 X PCR buffer (10 mM Tris-HCl pH 9.0, 0.1% triton X-100), 2 μL 25 mM $MgCl_2$, 5 μL of 1 mM dNTPs, 1 μL of 20 μM ACS289 primer (SEQ. ID. NO. 5), 1 μL of 20 μM ACS885 primer (table 2), 12.8 μL H_2O, and 0.3 μL Tag DNA polymerase (5 u/μL) (Promega Corporation, Madison, WI). The PCR was performed using 35 cycles of 94°C for 1 minute, 45°C for 1 minute, and 72°C for 2 minutes. Five μL of this reaction was electrophoresed in a 1.5% agarose gel as described above. A single product of approximately 603 bp was observed. Eighty μL of

sterile water, 10 μL of 3 M sodium acetate (pH 5.2), and 220 μL of 100% ethanol was added to the remainder of the reaction. After incubation at -20°C overnight, the DNA was recovered by centrifugation at 4°C for 30 minutes at 14,000 rpm. The DNA was washed with 400 μL of ice-cold 75% ethanol and resuspended in 25 μL of sterile water. The DNA concentration was determined to be 10 ng/μL using the ethidium bromide plate assay.

i) Labeling Coffee Fruit-Specific ACC Synthase DNA

A random primed probe was produced using the PCR-generated ACC synthase DNA and the Prime-a-Gene Kit (Promega Corporation, Madison, WI). Two and one-half μL of the DNA (25 ng) was added to 27.5 μL of sterile water and the DNA was denatured by boiling for 5 min. Ten μL of 5 X labeling buffer, 2 μL of unlabeled dNTP's [20 μM each; dCTP, dGTP, dTTP], 2 μL 1 mg/mL acetylated BSA, 1 μL 5u/μL *E. coli* DNA polymerase I Klenow fragment and 5 μL (50 μCi) of [α-^{32}P]dATP (3,000 Ci/mmole) (Dupont-NEN) were added to give a final volume of 50 μL. After 1 hr at room temperature, the reaction was terminated by the addition of 2 μL of 0.5 M Na_2EDTA and boiling for 2 min.

j) Screening of amplified library with the ACC synthase-specific probe.

Plaque lifts of the four 150x15 mm NZY plates containing 50,000 recombinant clones each were prepared. Four 132 mm Magna nylon transfer membranes (Micron Separations, Incorporated, Westborough, MA) were wetted by placing them on chromatography paper saturated with 5 X SSC buffer for approximately 10 sec. The membranes were placed on the plates containing the recombinant plaques for 5 min, removed and incubated, phage containing side up, for 2 min on chromatography paper saturated with 0.5 M NaOH and 1.5 M NaCl. The membranes were then neutralized by transferring onto chromatography paper saturated with 0.5 M tris-HCl (pH 8.0) and 1.5 M NaCl, for 5 min. After a brief 20 sec treatment on chromatography sheets saturated with 2 X SCC containing 0.2 M tris-hcl (pH 7.5), the filters were blotted dry. After 1 hour of air drying, DNA was cross-linked to the membranes by treatment with 12,000 μJoules of a 260 nm UV light in a UV Stratalinker 1800 (Stratagene, La Jolla, CA).

The four membranes were prehybridized at 65°C for 2 hours in 100 mL 6 X SSPE (52.2 g/L NaCl, 8.3 g/L $NaH_2PO_4.H_2O$, 2.2 g/L Na_2EDTA, [pH 7.4]), 5 X Denhardt's solution (1 g/L Ficoll, 1 g/L polyvinylpyrrolidone, 1 g/L BSA [pentax fraction V]), 0.5% SDS and 100 μg/mL denatured herring sperm

DNA in a Hybaid Mark II hybridization oven (National Labnet Company, Woodbridge, NJ) using HB-OV-BL bottles.

Hybridization was carried out at 65°C for 12 hours in 10 mL of 6 X SSPE containing 0.5% SDS, 100 μg/mL denatured herring sperm DNA, and 52 μL of the random primed probe described above. At the end of the hybridization period the hybridization solution was removed and the membranes were briefly washed with 100 mL of 2 X SSC containing 0.5% SDS at 65°C. They were then washed for an additional 30 min with the same amount of fresh buffer again at 65°C. The membranes were washed twice more for 30 min at 65°C with 100 mL of 0.2 X SSC containing 0.5% SDS, wrapped in a cellophane envelope and exposed to pre-flashed Fuji RX_{GCU} X-ray film at -70°C for 24 hours. Ten positive clones were obtained. The region of the original plates corresponding to the identified plaques were removed and placed in 1 mL of SM buffer containing 20 μL chloroform. Of these ten, 5 were re-plated at lower densities and rescreened as above to obtain individual plaques.

k) Characterization of Coffee-Fruit ACC synthase cDNA clones.

The size of the putative coffee ACC synthase cDNA clones was determined by polymerase chain reaction using primers homologous to a portion of

the T3 and T7 promoters present in the cloning vector and flanking the cDNA insertion site. The sequence of the primers are:

T3: 5'-TAATACGACTCACTATAGGG-3' (SEQ. ID NO. 6)

T7: 5'-AATTAACCCTCACTAAAGGG-3' (SEQ. ID NO. 7)

The conditions for PCR were as described above except that the temperature cycle was 95°C for 1 min., 50°C for 1 min. and 72°C for 2 min. Analysis was by agarose gel electrophoresis as before.

The three largest clones were recovered as phagemids by *in vivo* excision. Two hundred μL of phage stock from a single plaque was mixed with 200 μL of *E. coli* XL1-Blue MRF' grown to a density at $O.D._{600}$ of 1.0. One μL of ExAssist (Stratagene, La Jolla, CA) helper phage (>1 X 10^6 pfu/μL) was added and the tubes were incubated at 37°C for 15 min. Three mL of sterile LB broth were added and they were incubated for 3 hours at 37°C with shaking. After heating at 70°C for 20 min and centrifugation at 1,000 X *g* for 15 min, 1 mL of the supernatant, containing the excised pBluescript phagemid packaged as filamentous phage particles, was transferred to a sterile 1.5 mL microcentrifuge tube and stored at 4°C. Phagemids were recovered by adding 25 μL of the stock solution to 200 μL of *E. coli* Solar cells (Stratagene, La Jolla, CA) grown to a density of 1

when measured at $O.D._{600}$. After incubation at 37°C for 15 min, 200 μL of the cell mixture was plated on 100 X 15 mm NZY agar plates containing 50 μg/mL ampicillin. The plates were incubated overnight at 37°C. Individual colonies were picked into 10 mL of LB broth containing 50 μg/mL ampicillin and grown overnight in a 37°C shaking incubator. The cells were concentrated in a 1.5 mL sterile microcentrifuge tube by repeated centrifugation and the phasmid DNA was purified using the plasmid mini kit from QIAGEN. The bacterial pellets were washed with water and resuspended in 0.3 mL of buffer P1. Next, 0.3 mL of alkaline lysis buffer P2 was added, mixed gently, and incubated for less than 5 min at room temperature. Following the addition of 0.3 mL of chilled buffer P3 and mixing by inverting the tubes 6 times, the extracts were incubated on ice for 10 min and centrifuged at 14,000 rpm for 15 min in a microcentrifuge. The supernatants were removed and applied to QIAGEN-tip 20 columns that had been previously equilibrated with 1 mL of QDT buffer. The extracts were allowed to enter the resin of the columns by gravity flow. Once the flow had stopped, the columns were washed 4 times with 1 mL buffer QC. The DNAs were eluted by washing the QIAGEN-tip 20 columns with 0.8 mL buffer QF which was collected

into 1.5 mL microcentrifuge tubes. The DNA was precipitated by the addition of 0.7 volumes (560 μL) of isopropanol. The tubes were immediately centrifuged at 14,000 rpm for 30 min and the supernatant carefully removed. The pellets, containing the DNA, were washed 20 with 1 mL of ice-cold 70% ethanol, centrifuged as above, and air dried for 5 min. The DNA was resuspended in 50 μL sterile H_2O. The concentration of DNA from one plasmid isolation was 0.1 μg/μL by fluormetric analysis.

Sequencing reactions were performed by mixing 8 μL of phagmid DNA (0.8 μg) with 4 μL of either T3 or T7 sequencing primers (0.8 pmol/μL). Automated DNA sequencing was carried out on these samples at the University of Hawaii Biotechnology Service Center. About 350 bp of sequence from both the 5' and the 3' end of the cDNA was obtained. New sequencing primers were synthesized based on sequences near the end of the previous sequences and used in the same manner to complete the sequence of both strands of the cDNA. The complete sequence of the coffee fruit-expressed ACC synthase cDNA is given in Figure 1. The deduced amino acid sequence of the coffee fruit-expressed ACC synthase is given in Figure 2.

The sequence of the coffee ACC synthase cDNA clone and deduced protein was compared with other ACC synthase genes present in GenBank. The cDNA isolated from coffee fruit shows from 68.3% to 58.1% identity to other ACC synthases present in GenBank. And, the protein sequence deduced from this cDNA shows from 67.9% to 50.5% identity to other ACC synthases. However, this cDNA is unique in that no other sequence greater than 1500 bp showed greater than 68.3% identity to it.

EXAMPLE 2

Isolation of Coffee Fruit-Specific ACC Oxidase

a) Synthesis of ACC Oxidase specific oligonucleotide primers.

The isolation of total RNA, mRNA, and the synthesis of coffee fruit-specific cDNA was as described above.

Twelve ACC oxidase sequences, obtained from GenBank, were aligned using the Pileup program of GCG (Genetics Computer Group, Madison, WI). A region approximately 1000 bp from the translation start codon was found to be conserved and a degenerate oligonucleotide primer

5'-TCATIGCKKCRAKIGGTTC-3' (SEQ. ID NO. 8)

corresponding to this region was synthesized. Inosine (I) was placed at positions showing no sequence conservation, since position could be any

of A, T, G or C. Positions showing two-fold ambiguity were prepared with mixed residues (T/G or A/G). We also prepared a second primer homologous to a region of the papaya fruit-expressed ACC oxidase cDNA that had been previously cloned in our laboratory and situated approximately 372 bp from the translational start codon:

5'-GACACTGTGGAGAGGCTGAC-3' (SEQ. ID NO. 9)

The two primers were used in a PCR reaction to amplify a portion of the coffee fruit-expressed ACC oxidase. The PCR contained 0.2 μL (10 ng) cDNA fraction 3 (described in Example 1), 5 μL 10 X PCR buffer, 3 μL 25 mM $MgCl_2$, 1 μL of each of the four 10 mM dNTPs, 1 μL of a 20 μM solution of each primer, 0.3 μL Taq DNA polymerase (promega Corporation, Madison, WI) and 38.5 μL water. PCR conditions were 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. A 5 min incubation at 72°C was carried out after the last cycle. A 20 μL aliquot of the product was electrophoresed in a 1.5% agarose gel as described previously and revealed an approximately 800 bp product. The DNA was excised from the gel and mixed with 200 μL of sterile water in a 1.5 mL microcentrifuge tube. After boiling for 5 min, 2 μL was used as a template in a 50 μL PCR reaction as above using the same primers. Gel

electrophoresis performed as described above using 20 μL of the PCR reaction indicated the presence of a single 800 bp product. To the remaining 30 μL of the PCR reaction 20 μL chloroform and 100 μL water was added. The contents were mixed and centrifuged for 2 minutes at 14,000 rpm in a microcentrifuge. The upper aqueous phase containing the DNA was removed to a clean microcentrifuge tube. A portion of this DNA was radioactively labeled by random primed synthesis as described above.

b) Screening of amplified library with random primed probe.

The amplified coffee-fruit cDNA described in Example 1 was used to prepare four 150 X 10 mm NZY plates as previously described. Prehybridization, hybridization and recovery of clones was as previously described except that the ACC oxidase sequence obtained by PCR was used as the probe.

c) Characterization of Coffee-Fruit ACC-oxidase cDNA clones.

The size of the coffee ACC-oxidase cDNA clones was determined by polymerase chain reaction using primers homologous to the T3 and T7 promoters as described in Example 1.

The sequence of the largest coffee ACC oxidase cDNA clone was obtained as described in Example 1 and compared with ACC oxidase genes present in

GenBank. Figure 3 gives the sequence of the coffee fruit-specific ACC oxidase. Figure 4 gives the deduced amino acid sequence of this protein. The cDNA was determined to encode ACC oxidase because it is from 50.4% to 82.5% identical to other ACC synthases nucleic acid sequenccs present in GenBank. Also, the deduced protein sequence is from 32.5% to 86.5% identical to other ACC oxidases.

The foregoing examples are for illustrative purposes only, and should not be viewed as limiting the scope of applicants' invention, which is set forth in the claims appended hereto.

SEQUENCE LISTING 37

(1) GENERAL INFORMATION:

(i) APPLICANT: STILES, JOHN I.
MOISYADI, ISTEFO
NEUPANE, KABI R.

(ii) TITLE OF INVENTION: PURIFIED PROTEINS, RECOMBINANT DNA SEQUENCES AND PROCESSES FOR CONTROLLING THE RIPENING OF COFFEE

(iii) NUMBER OF SEQUENCES: 13

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: JONES, DAY, REAVIS & POGUE
(B) STREET: NORTH POINT, 901 LAKESIDE AVENUE
(C) CITY: CLEVELAND
(D) STATE: OHIO
(E) COUNTRY: USA
(F) ZIP: 44114

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette, 3.5 inch, 1.44 Mb storage
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: MS-DOS v. 5.1
(D) SOFTWARE: WordPerfect v. 6.1

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: 08/695,412
(B) FILING DATE: 12-AUG-1996
(C) CLASSIFICATION: 435

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(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: GRIFFITH, CALVIN P.
(B) REGISTRATION NUMBER: 34,831
(C) REFERENCE/DOCKET NUMBER: 265036600002

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (216) 586-7050
(B) TELEFAX: (216) 579-0212

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acid residues
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Fragment A
(B) LOCATION: 17..1480

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ile Asn Tyr Ala Ser Gly Ala Ser Gly Ile Leu Asp Gln Xaa Gly
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acid residues
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ile Asn Tyr Ala Ser Gly Ala Ser Gly Ile Leu Asp Gln Thr
1 5 10 14

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: OTHER NUCLEIC ACID
(A) DESCRIPTION: PRIMER

(v) FRAGMENT TYPE: Internal

(ix) FEATURE:
(A) OTHER INFORMATION: N IS INOSINE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATNAAYTAYG CNAGYGGNGC 20

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: OTHER NUCLEAR ACID
(A) DESCRIPTION: PRIMER

(v) FRAGMENT TYPE: INTERNAL

(ix) FEATURE
(A) OTHER INFORMATION: N IS INOSINE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATNAAYTAYG CNAGYGGNGC 20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: OTHER NUCLEIC ACID
(A) DESCRIPTION: PRIMER

(v) FRAGMENT TYPE: INTERNAL

(ix) FEATURE
(A) OTHER INFORMATION: N IS INOSINE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGNCCAGNCG NYTAYTTNAT 20

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
(A) DESCRIPTION: PRIMER

(v) FRAGMENT TYPE: INTERNAL

(ix) FEATURE
(A) OTHER INFORMATION: N IS INOSINE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGNCCYCTYG CYTAYTTNAT 20

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acid residues
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: INTERNAL

(ix) FEATURE
(D) OTHER INFORMATION: Xaa is either Thr or Asp

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Gln Tyr Val Pro Cys Tyr Phe Xaa Phe Ile Asp Asp Gln Asp
1 5 10 14

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: OTHER NUCLEIC ACID
(A) DESCRIPTION: PRIMER

(v) FRAGMENT TYPE: Internal

(ix) FEATURE
(A) OTHER INFORMATION: N IS INOSINE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CAWTATGTNC CNTGTTATTT 20

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: OTHER NUCLEIC ACID
(A) DESCRIPTION: PRIMER

(v) FRAGMENT TYPE: Internal

(ix) FEATURE
(A) OTHER INFORMATION: N IS INOSINE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AAWTAWCAHG GNACWTATTG 20

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 488 amino acid residues
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 178..1653

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Met Glu Phe Ser Leu Lys Asn Glu Gln Gln Gln Leu Leu Ser Lys
1 5 10 15

Met Ala Thr Asn Asp Gly His Gly Glu Asn Ser Pro Tyr Phe Asp
20 25 30

Gly Trp Lys Ala Tyr Asp Ser Asp Pro Tyr His Pro Thr Arg Asn
35 40 45

Pro Asn Gly Val Ile Gln Met Gly Leu Ala Glu Asn Gln Leu Cys
50 55 60

Phe Asp Leu Ile Glu Glu Trp Val Leu Asn Asn Pro Glu Ala Ser
65 70 75

Ile Cys Thr Ala Glu Gly Ala Asn Lys Phe Met Glu Val Ala Ile
80 85 90

Tyr Gln Asp Tyr His Gly Leu Pro Glu Phe Arg Asn Ala Val Ala
95 100 105

Arg Phe Met Glu Lys Val Arg Gly Asp Arg Val Lys Phe Asp Pro
110 115 120

Asn Arg Ile Val Met Ser Gly Gly Ala Thr Gly Ala His Glu Thr
125 130 135

Leu Ala Phe Cys Leu Ala Asp Pro Glu Asp Ala Phe Leu Val Pro
140 145 150

Thr Pro Tyr Tyr Pro Gly Phe Asp Arg Asp Leu Arg Trp Arg Thr
155 160 165

Gly Met Gln Leu Leu Pro Ile Val Cys Arg Ser Ser Asn Asp Phe
170 175 180

Lys Val Thr Lys Glu Ser Met Glu Ala Ala Tyr Gln Lys Ala Gln
185 190 195

Glu Ala Asn Ile Arg Val Lys Gly Phe Leu Leu Asn Asn Pro Ser
200 205 210

Asn Pro Leu Gly Thr Val Leu Asp Arg Glu Thr Leu Ile Asp Ile
215 220 225

Val Thr Phe Ile Asn Asp Lys Asn Ile His Leu Ile Cys Asp Glu
230 235 240

Ile Tyr Ser Ala Thr Val Phe Ser Gln Pro Glu Phe Ile Ser Ile
245 250 255

Ser Glu Ile Ile Glu His Asp Val Gln Cys Asn Arg Asp Leu Ile
260 265 270

His Leu Val Tyr Ser Leu Ser Lys Asp Leu Gly Phe Pro Gly Phe
275 280 285

Arg Val Gly Ile Leu Tyr Ser Tyr Asn Asp Ala Val Val Ser Cys
290 295 300

Ala Arg Lys Met Ser Ser Phe Gly Leu Val Ser Thr Gln Thr Gln
305 310 315

His Leu Ile Ala Ser Met Leu Ser Asp Glu Ala Phe Met Asp Lys
320 325 330

Ile Ile Ser Thr Ser Ser Glu Arg Leu Ala Ala Arg His Gly Leu
335 340 345

Phe Thr Arg Gly Leu Ala Gln Val Gly Ile Gly Thr Leu Lys Ser
350 355 360

Ser Ala Gly Leu Tyr Phe Trp Met Asp Leu Arg Arg Leu Leu Arg
365 370 375

Glu Ser Thr Phe Glu Ala Glu Met Glu Leu Trp Arg Ile Ile Ile
380 385 390

His Glu Val Lys Leu Asn Val Ser Pro Gly Leu Ser Phe His Cys
395 400 405

Ser Glu Pro Gly Trp Phe Arg Val Cys Phe Ala Asn Met Asp Asp
410 415 420

Glu Ser Val Arg Val Ala Leu Arg Arg Ile His Lys Phe Val Leu
425 430 435

Val Gln Gly Lys Ala Thr Glu Pro Thr Thr Pro Lys Ser Arg Cys
440 445 450

Gly Ser Ser Lys Leu Gln Leu Ser Leu Ser Phe Arg Arg Leu Asp
455 460 465

Glu Arg Val Met Gly Ser His Met Met Ser Pro His Ser Pro Met
470 475 480

Ala Ser Pro Leu Val Arg Ala Thr
485

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2040 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(ix) Feature:

(A) NAME/KEY: CDS

(B) LOCATION: 178..1653

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTAATCTCTT CTAAAATCAA CCATTCTCTT CATTCTTCAC TTGACAAGGC 50

CACTGCATTC TTCATTCTTT CTTGATATAT AGCCATTTTT TTCATTCTTT 100

CTTGATATAT AGCCATTTTT TTCATTCTTT CTTCATTCAT TGTCTGGAGA 150

AGTTGGTTGA GTTTTCTTGA AAATTCAAGC AAAACA ATG GAG TTC AGT 198
Met Glu Phe Ser
1

TTG AAA AAC GAA CAA CAA CAA CTC TTG TCG AAG ATG GCA ACC 240
Leu Lys Asn Glu Gln Gln Gln Leu Leu Ser Lys Met Ala Thr
5 10 15

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AAC GAT GGA CAT GGC GAA AAC TCG CCT TAT TTT GAT GGT TGG     282
Asn Asp Gly His Gly Glu Asn Ser Pro Tyr Phe Asp Gly Trp
     20                  25                  30

AAG GCA TAT GAT AGT GAT CCT TAC CAT CCC ACC AGA AAT CCT     324
Lys Ala Tyr Asp Ser Asp Pro Tyr His Pro Thr Arg Asn Pro
         35                  40                  45

AAT GGT GTT ATA CAG ATG GGA CTC GCA GAA AAT CAG TTA TGC     366
Asn Gly Val Ile Gln Met Gly Leu Ala Glu Asn Gln Leu Cys
             50                  55                  60

TTT GAT TTG ATC GAG GAA TGG GTT CTG AAC AAT CCA GAG GCT     408
Phe Asp Leu Ile Glu Glu Trp Val Leu Asn Asn Pro Glu Ala
                 65                  70

TCC ATT TGC ACA GCA GAA GGA GCG AAC AAA TTC ATG GAA GTT     450
Ser Ile Cys Thr Ala Glu Gly Ala Asn Lys Phe Met Glu Val
 75                  80                  85

GCT ATC TAT CAA GAT TAT CAT GGC TTG CCA GAG TTC AGA AAT     492
Ala Ile Tyr Gln Asp Tyr His Gly Leu Pro Glu Phe Arg Asn
     90                  95                 100

GCT GTA GCA AGG TTC ATG GAG AAG GTG AGA GGT GAC AGA GTC     534
Ala Val Ala Arg Phe Met Glu Lys Val Arg Gly Asp Arg Val
        105                 110                 115

AAG TTC GAT CCC AAC CGC ATT GTG ATG AGT GGT GGG GCA ACC     576
Lys Phe Asp Pro Asn Arg Ile Val Met Ser Gly Gly Ala Thr
            120                 125                 130

GGA GCT CAT GAA ACT CTG GCC TTC TGT TTA GCT GAC CCT GAA     618
Gly Ala His Glu Thr Leu Ala Phe Cys Leu Ala Asp Pro Glu
                135                 140

GAT GCG TTT TTG GTA CCC ACA CCA TAT TAT CCA GGA TTT GAT     660
Asp Ala Phe Leu Val Pro Thr Pro Tyr Tyr Pro Gly Phe Asp
145                 150                 155

CGG GAT TTG AGG TGG CGA ACA GGG ATG CAA CTT CTT CCA ATT     702
Arg Asp Leu Arg Trp Arg Thr Gly Met Gln Leu Leu Pro Ile
    160                 165                 170
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GTT TGT CGC AGC TCC AAT GAT TTT AAG GTC ACT AAA GAA TCC     744
Val Cys Arg Ser Ser Asn Asp Phe Lys Val Thr Lys Glu Ser
        175                 180                 185

ATG GAA GCT GCT TAT CAG AAA GCT CAA GAA GCC AAC ATC AGA     786
Met Glu Ala Ala Tyr Gln Lys Ala Gln Glu Ala Asn Ile Arg
            190                 195                 200

GTA AAG GGG TTC CTC TTA AAT AAT CCA TCA AAT CCA TTG GGA     828
Val Lys Gly Phe Leu Leu Asn Asn Pro Ser Asn Pro Leu Gly
                205                 210

ACT GTT CTT GAC AGG GAA ACT TTG ATT GAT ATA GTC ACA TTC     870
Thr Val Leu Asp Arg Glu Thr Leu Ile Asp Ile Val Thr Phe
215                 220                 225

ATC AAT GAC AAA AAT ATC CAC TTG ATT TGT GAT GAG ATA TAT     912
Ile Asn Asp Lys Asn Ile His Leu Ile Cys Asp Glu Ile Tyr
    230                 235                 240

TCT GCC ACC GTC TTC AGC CAG CCC GAA TTC ATC AGC ATC TCT     954
Ser Ala Thr Val Phe Ser Gln Pro Glu Phe Ile Ser Ile Ser
        245                 250                 255

GAA ATA ATT GAG CAT GAT GTT CAA TGC AAC CGT GAT CTC ATA     996
Glu Ile Ile Glu His Asp Val Gln Cys Asn Arg Asp Leu Ile
            260                 265                 270

CAT CTT GTG TAT AGC CTG TCC AAG GAC TTG GGC TTC CCT GGA    1038
His Leu Val Tyr Ser Leu Ser Lys Asp Leu Gly Phe Pro Gly
                275                 280

TTC AGA GTT GGC ATT TTG TAT TCA TAT AAT GAC GCT GTT GTC    1080
Phe Arg Val Gly Ile Leu Tyr Ser Tyr Asn Asp Ala Val Val
285                 290                 295

AGC TGT GCT AGA AAA ATG TCG AGT TTC GGC CTT GTT TCA ACA    1122
Ser Cys Ala Arg Lys Met Ser Ser Phe Gly Leu Val Ser Thr
    300                 305                 310

CAA ACT CAG CAT CTG ATT GCA TCA ATG TTA TCG GAC GAA GCA    1164
Gln Thr Gln His Leu Ile Ala Ser Met Leu Ser Asp Glu Ala
        315                 320                 325
```

```
TTT ATG GAC AAA ATC ATT TCC ACG AGC TCA GAG AGA TTA GCT   1206
Phe Met Asp Lys Ile Ile Ser Thr Ser Ser Glu Arg Leu Ala
            330                 335                 340

GCA AGG CAT GGT CTT TTC ACA AGA GGA CTT GCT CAA GTA GGC   1248
Ala Arg His Gly Leu Phe Thr Arg Gly Leu Ala Gln Val Gly
                345                 350

ATT GGC ACC TTA AAA AGC AGT GCG GGC CTT TAT TTC TGG ATG   1290
Ile Gly Thr Leu Lys Ser Ser Ala Gly Leu Tyr Phe Trp Met
355                 360                 365

GAC TTA AGG AGA CTC CTC AGG GAG TCC ACA TTT GAG GCA GAA   1332
Asp Leu Arg Arg Leu Leu Arg Glu Ser Thr Phe Glu Ala Glu
    370                 375                 380

ATG GAA CTT TGG AGG ATC ATA ATA CAT GAA GTC AAG CTC AAT   1374
Met Glu Leu Trp Arg Ile Ile Ile His Glu Val Lys Leu Asn
        385                 390                 395

GTT TCA CCA GGC TTA TCT TTC CAT TGC TCA GAA CCA GGA TGG   1416
Val Ser Pro Gly Leu Ser Phe His Cys Ser Glu Pro Gly Trp
            400                 405                 410

TTC AGA GTT TGC TTT GCC AAC ATG GAC GAC GAA AGT GTG AGA   1458
Phe Arg Val Cys Phe Ala Asn Met Asp Asp Glu Ser Val Arg
                415                 420

GTT GCT CTC AGA AGA ATC CAC AAA TTT GTG CTT GTT CAG GGC   1500
Val Ala Leu Arg Arg Ile His Lys Phe Val Leu Val Gln Gly
425                 430                 435

AAG GCA ACA GAG CCA ACA ACT CCA AAG AGT CGC TGC GGA AGC   1542
Lys Ala Thr Glu Pro Thr Thr Pro Lys Ser Arg Cys Gly Ser
    440                 445                 450

AGC AAA CTT CAA CTC AGC TTA TCT TTC CGC AGA TTG GAC GAA   1584
Ser Lys Leu Gln Leu Ser Leu Ser Phe Arg Arg Leu Asp Glu
        455                 460                 465

AGG GTG ATG GGA TCG CAT ATG ATG TCC CCT CAC TCC CCG ATG   1626
Arg Val Met Gly Ser His Met Met Ser Pro His Ser Pro Met
            470                 475                 480
```

```
GCT TCA CCT TTG GTT CGG GCT ACA TAAATCATTT CTTGATCAGA      1670
Ala Ser Pro Leu Val Arg Ala Thr
                485

TCATATAGCA AAGATTCCTG AGTAAATACT CGAAACCCTT TCTGGATAAC     1720

TGAAAAGAGA GTTGTTGATT CTTTGCTGTA TCATACAAAC ACGTTACAGG     1770

CATTTTTTGG CCATCTGATG CGTGCAAATT GCATCAAATG CTTTTATTAT     1820

TGTCATATTC ATTTGTGTAC CTTGGTTTTC CTTGCCCTTC AGTCCTCCTT     1870

GTTTTTTGTT TCTTTGTTAT TATTTTCTTC CAGTTGATCA GTTAAACGAA     1920

GGAAGCTCAA TTGTTTCAAG CTATTAGTAA CAGATCATTT TGTAATAGCA     1970

ATAGTTTCAG GATTCTGAAA TGAAAGTTTA TCATTTTTCC ATCATTTTAA     2020

AAAAAAAAAA AAAAAAAAAA                                     2040
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 318 amino acid residues
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 46..1003

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12

```
Met Ala Thr Phe Pro Leu Ile Asp Met Glu Lys Leu Asp Gly Glu
 1               5                  10                  15

Glu Arg Ala Ala Thr Met Gly Val Ile Lys Asp Ala Cys Glu Ser
            20                  25                  30

Trp Gly Phe Phe Glu Val Leu Asn His Gly Ile Ser Asn Glu Leu
        35                  40                  45

Met Asp Thr Val Glu Arg Leu Thr Lys Glu His Tyr Lys Lys Cys
    50                  55                  60
```

Met Glu Leu Lys Phe Lys Glu Met Val Glu Ser Lys Glu Leu Glu
65 70 75

Ala Val Gln Thr Glu Ile Asn Asp Leu Asp Trp Glu Ser Thr Phe
80 85 90

Phe Leu Arg His Leu Pro Val Ser Asn Ile Ser Glu Val Pro Asp
95 100 105

Leu Asp Asp Glu Tyr Arg Lys Val Met Lys Glu Phe Ala Leu Gln
110 115 120

Leu Glu Lys Leu Ala Glu Leu Leu Leu Asp Leu Leu Cys Glu Asn
125 130 135

Leu Gly Leu Glu Lys Gly Tyr Leu Lys Lys Ala Phe Tyr Gly Thr
140 145 150

Lys Gly Pro Thr Phe Gly Thr Lys Val Ser Asn Tyr Pro Pro Cys
155 160 165

Pro Arg Pro Glu Leu Ile Lys Gly Leu Arg Ala His Thr Asp Ala
170 175 180

Gly Gly Ile Ile Leu Leu Phe Gln Asp Asp Lys Val Ser Gly Leu
185 190 195

Gln Leu Leu Lys Asp Gly Glu Trp Val Asp Val Pro Pro Met Arg
200 205 210

His Ser Ile Val Ile Asn Ile Gly Asp Gln Leu Glu Val Ile Thr
215 220 225

Asn Gly Lys Tyr Lys Ser Val Met His Arg Val Ile Ala Gln Pro
230 235 240

Asp Gly Asn Arg Met Ser Leu Ala Ser Phe Tyr Asn Pro Gly Ser
245 250 255

Asp Ala Val Ile Tyr Pro Ala Pro Ala Leu Val Glu Lys Glu Ala
260 265 270

Glu Asp Lys Gln Ile Tyr Pro Lys Phe Val Phe Glu Asp Tyr Met
275 280 285

Lys Leu Tyr Ala Gly Leu Lys Phe Gln Ala Lys Glu Pro Arg Phe
290 295 300

Glu Ala Met Lys Ala Val Glu Ser Thr Val Asn Leu Gly Pro Ile
305 310 315

Ala Thr Val
318

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1320 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(ix) Feature:

(A) NAME/KEY: CDS

(B) LOCATION: 46..1003

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
TGTAAACGAA GCATAAGCAC AAGCAAACAC AAACTAGAAA GAGAG ATG          48
                                                  Met
                                                   1

GCT ACA TTC CCC CTA ATC GAC ATG GAG AAG CTT GAC GGT GAA         90
Ala Thr Phe Pro Leu Ile Asp Met Glu Lys Leu Asp Gly Glu
              5                  10                  15

GAG AGG GCT GCC ACT ATG GGA GTC ATA AAA GAT GCT TGT GAA        132
Glu Arg Ala Ala Thr Met Gly Val Ile Lys Asp Ala Cys Glu
                  20                  25

AGC TGG GGC TTC TTT GAG GTG TTG AAT CAT GGG ATA TCT AAT        174
Ser Trp Gly Phe Phe Glu Val Leu Asn His Gly Ile Ser Asn
30                  35                  40

GAG CTC ATG GAC ACA GTG GAG AGG CTA ACA AAG GAG CAT TAC        216
Glu Leu Met Asp Thr Val Glu Arg Leu Thr Lys Glu His Tyr
     45                  50                  55

AAG AAA TGT ATG GAA CTA AAG TTC AAG GAA ATG GTG GAG AGC        258
Lys Lys Cys Met Glu Leu Lys Phe Lys Glu Met Val Glu Ser
         60                  65                  70

AAG GAA TTG GAA GCT GTT CAG ACT GAG ATC AAT GAT TTG GAC        300
Lys Glu Leu Glu Ala Val Gln Thr Glu Ile Asn Asp Leu Asp
              75                  80                  85
```

CLAIMS

1. A substantially pure ACC synthase from *Coffea arabica* consisting essentially of the amino acid sequence: (SEQ. ID. NO. 10)

2. Substantially pure nucleic acid sequence that codes on expression for the ACC synthase produced by *Coffea arabica* comprising: (SEQ. ID. NO. 11)

3. The substantially pure nucleic acid sequence that codes on expression for the ACC synthase produced by *Coffea arabica* of claim 2, wherein the nucleic acid sequence is limited to the coding regions of SEQ. ID. No. 11.

4. A method for controlling the ripening of *Coffea arabica* comprising:

a) transforming coffee plants with a DNA sequence that is antisense to the DNA sequence SEQ. ID. NO. 11;

b) growing plants transformed with the DNA sequence of a) above; and

c) applying exogenous ethylene to the transformed plants after coffee fruit has matured.

5. The method for controlling the ripening of *Coffea arabica* of claim 4, wherein the DNA sequence used for transforming is limited to a sequence that is antisense to the coding region of SEQ. ID. No. 11.

6. The method for controlling fruit ripening of claims 4 or 5, wherein gaseous ethylene is applied to the entire plant, to cause ripening of substantially all of the fruit simultaneously.

7. A substantially pure ACC oxidase from *Coffea arabica* consisting essentially of the amino acid sequence: (SEQ. ID. NO. 12).

8. Substantially pure nucleic acid sequence that codes an expression for *Coffee arabica* ACC oxidase comprising: (SEQ. ID. No. 13).

9. The substantially pure nucleic acid sequence that codes on expression for ACC oxidase produced by *Coffee arabica* of claim 8, wherein the nucleic acid sequence is limited to the coding regions of SEQ. ID. No. 13.

10. A method for controlling ripening of *Coffea arabica* comprising:

a) transforming coffee plants with a DNA sequence that is antisense to the DNA sequence: (SEQ. ID. No. 13);

b) growing plants transformed with the DNA sequence of a) above; and

c) applying exogenous ethylene to the transformed plants after coffee fruit has matured.

11. The method for controlling ripening of *Coffea arabica* of claim 10, wherein the DNA sequence used for transforming is limited to a sequence that is antisense to the coding region of SEQ. ID. NO. 13.

12. The method for controlling fruit ripening of claims 9 and 10, wherein the gaseous ethylene is applied to the entire plant, to cause ripening of substantially all of the fruit simultaneously.

13. A coffee plant having suppressed expression of ACC synthase.

14. A coffee plant having suppressed expression of ACC oxidase.

15. A coffee plant having suppressed expression of ACC synthase and suppressed expression of ACC oxidase.

16. A coffee plant comprising a DNA sequence that codes on transcription for a mRNA that is antisense to the mRNA that codes on expression for ACC synthase.

17. A coffee fruit from the coffee plant of claim 16.

18. A coffee bean from the coffee plant of claim 16.

19. A coffee plant comprising a DNA sequence that codes on transcription for a mRNA that is antisense to the mRNA that codes on expression for ACC oxidase.

20. A coffee fruit from the coffee plant of claim 19.

21. A coffee bean from the coffee plant of claim 19.

22. A coffee plant comprising (i) a first DNA sequence that codes on transcription for a mRNA that is antisense to the mRNA that codes on expression for ACC synthase, and (ii) a second DNA sequence that codes on transcription for a mRNA that is antisense to the mRNA that codes on expression for ACC oxidase.

23. A coffee fruit from the coffee plant of claim 22.

24. A coffee bean from the coffee plant of claim 22.

25. A coffee plant comprising a DNA sequence that is antisense to all or part of the DNA sequence specified in SEQ ID NO:11.

26. A coffee plant comprising a DNA sequence that is antisense to all or part of the DNA sequence specified in SEQ ID NO:13.

27. A coffee plant comprising (i) a first DNA sequence that is antisense to all or part of the DNA

sequence specified in SEQ ID NO:11, and (ii) a second DNA sequence that is antisense to all or part of the DNA sequence specified in SEQ ID NO:13.

28. A coffee plant produced by the process of inserting into the plant genome a DNA sequence that codes on transcription for a mRNA that is antisense to the mRNA that codes on expression for ACC synthase.

29. A coffee plant produced by the process of inserting into the plant genome a DNA sequence that codes on transcription for a mRNA that is antisense to the mRNA that codes on expression for ACC oxidase.

30. A coffee plant produced by the process of inserting into the plant genome (i) a DNA sequence that codes on transcription for a mRNA that is antisense to the mRNA that codes on expression for ACC synthase, and (ii) a DNA sequence that codes on transcription for a mRNA that is antisense to the mRNA that codes on expression for ACC oxidase.

31. A method for transforming a coffee plant with a DNA sequence that codes on transcription for a mRNA that is antisense to the mRNA that codes on expression for ACC synthase, comprising the steps of:

providing a transforming vector comprising a DNA sequence that codes on expression for ACC synthase, wherein the DNA sequence is inserted into the transforming vector in an inverted orientation; and

inserting the transforming vector into the tissue of the coffee plant, wherein the inverted DNA sequence thereafter becomes inserted into the genome of the coffee plant.

32. A method for transforming a coffee plant with a DNA sequence that codes on transcription for a mRNA that is antisense to the mRNA that codes on expression for ACC oxidase, comprising the steps of:

providing a transforming vector comprising a DNA sequence that codes on expression for ACC oxidase, wherein the DNA sequence is inserted into the transforming vector in an inverted orientation;

inserting the transforming vector into the tissue of the coffee plant, wherein the inverted DNA sequence thereafter becomes inserted into the genome of the coffee plant.

33. A method for transforming a coffee plant with (i) a first DNA sequence that codes on transcription for a mRNA that is antisense to the mRNA that codes on expression for ACC synthase, and (ii) a second DNA sequence that codes on transcription for a mRNA that is antisense to the mRNA that codes on expression for ACC oxidase, comprising the steps of:

providing a first transforming vector comprising a first DNA sequence that codes on expression for ACC synthase, wherein the first DNA sequence is inserted into the first transforming vector in an inverted orientation;

providing a second transforming vector comprising a second DNA sequence that codes on expression for ACC oxidase, wherein the second DNA sequence is inserted into the second transforming vector in an inverted orientation;

inserting the first transforming vector into the tissue of the coffee plant, wherein the first inverted DNA sequence thereafter

becomes inserted into the genome of the coffee plant; and

inserting the second transforming vector into the tissue of the coffee plant, wherein the second inverted DNA sequence thereafter becomes inserted into the genome of the coffee plant.

Fig 1: DEDUCED AMINO ACID SEQUENCE OF ACC SYNTHASE FROM *COFFEA ARABICA* (SEQ. ID. NO. 10)

```
Met Glu Phe Ser Leu Lys Asn Glu Gln Gln Gln Leu Leu Ser Lys
 1               5                  10                  15

Met Ala Thr Asn Asp Gly His Gly Glu Asn Ser Pro Tyr Phe Asp Gly
            20                  25                  30

Trp Lys Ala Tyr Asp Ser Asp Pro Tyr His Pro Thr Arg Asn Pro Asn
        35                  40                  45

Gly Val Ile Gln Met Gly Leu Ala Glu Asn Gln Leu Cys Phe Asp Leu
    50                  55                  60

Ile Glu Glu Trp Val Leu Asn Asn Pro Glu Ala Ser Ile Cys Thr Ala
65                  70                  75

Glu Gly Ala Asn Lys Phe Met Glu Val Ala Ile Tyr Gln Asp Tyr His
 80                  85                  90                  95

Gly Leu Pro Glu Phe Arg Asn Ala Val Ala Arg Phe Met Glu Lys Val
            100                 105                 110

Arg Gly Asp Arg Val Lys Phe Asp Pro Asn Arg Ile Val Met Ser Gly
        115                 120                 125
```

Gly Ala Thr Gly Ala His Glu Thr Leu Ala Phe Cys Leu Ala Asp Pro
130 135 140

Glu Asp Ala Phe Leu Val Pro Thr Pro Tyr Tyr Pro Gly Phe Asp Arg
145 150 155

Asp Leu Arg Trp Arg Thr Gly Met Gln Leu Leu Pro Ile Val Cys Arg
160 165 170 175

Ser Ser Asn Asp Phe Lys Val Thr Lys Glu Ser Met Glu Ala Ala Tyr
180 185 190

Gln Lys Ala Gln Glu Ala Asn Ile Arg Val Lys Gly Phe Leu Leu Asn
195 200 205

Asn Pro Ser Asn Pro Leu Gly Thr Val Leu Asp Arg Glu Thr Leu Ile
210 215 220

Asp Ile Val Thr Phe Ile Asn Asp Lys Asn Ile His Leu Ile Cys Asp
225 230 235

Glu Ile Tyr Ser Ala Thr Val Phe Ser Gln Pro Glu Phe Ile Ser Ile
240 245 250 255

Ser Glu Ile Ile Glu His Asp Val Gln Cys Asn Arg Asp Leu Ile His
260 265 270

```
Leu Val Tyr Ser Leu Ser Lys Asp Leu Gly Phe Pro Gly Phe Arg Val
            275                 280                 285

Gly Ile Leu Tyr Ser Tyr Asn Asp Ala Val Val Ser Cys Ala Arg Lys
        290                 295                 300
Met Ser Ser Phe Gly Leu Val Ser Thr Gln Thr Gln His Leu Ile Ala
    305                 310                 315

Ser Met Leu Ser Asp Glu Ala Phe Met Asp Lys Ile Ile Ser Thr Ser
320                 325                 330                 335

Ser Glu Arg Leu Ala Ala Arg His Gly Leu Phe Thr Arg Gly Leu Ala
                340                 345                 350

Gln Val Gly Ile Gly Thr Leu Lys Ser Ser Ala Gly Leu Tyr Phe Trp
            355                 360                 365

Met Asp Leu Arg Arg Leu Leu Arg Glu Ser Thr Phe Glu Ala Glu Met
        370                 375                 380

Glu Leu Trp Arg Ile Ile Ile His Glu Val Lys Leu Asn Val Ser Pro
    385                 390                 395

Gly Leu Ser Phe His Cys Ser Glu Pro Gly Trp Phe Arg Val Cys Phe
400                 405                 410                 415
```

Ala Asn Met Asp Asp Glu Ser Val Arg Val Ala Leu Arg Arg Ile His
420 425 430

Lys Phe Val Leu Val Gln Gly Lys Ala Thr Glu Pro Thr Thr Pro Lys
435 440 445

Ser Arg Cys Gly Ser Ser Lys Leu Gln Leu Ser Leu Ser Phe Arg Arg
450 455 460

Leu Asp Glu Arg Val Met Gly Ser His Met Met Ser Pro His Ser Pro
465 470 475

Met Ala Ser Pro Leu Val Arg Ala Thr
480 485

Fig 2.: Coffee fruit-expressed ACC synthase gene sequence. (SEQ. ID. NO. 11)

GTAATCTCTT CTAAAATCAA CCATTCTCTT CATTCTTCAC TTGACAAGGC CACTGCATTC 60

TTCATTCTTT CTTGATATAT AGCCATTTTT TTCATTCTTT CTTGATATAT AGCCATTTTT 120

TTCATTCTTT CTTCATTCAT TGTCTGGAGA AGTTGGTTGA GTTTTCTTGA AAATTCAAGC 180

AAAACA ATG GAG TTC AGT TTG AAA AAC GAA CAA CAA CAA CTC TTG TCG AAG 231

ATG GCA ACC AAC GAT GGA CAT GGC GAA AAC TCG CCT TAT TTT GAT GGT 279

TGG AAG GCA TAT GAT AGT GAT CCT TAC CAT CCC ACC AGA AAT CCT AAT 327

GGT GTT ATA CAG ATG GGA CTC GCA GAA AAT CAG TTA TGC TTT GAT TTG 375

ATC GAG GAA TGG GTT CTG AAC AAT CCA GAG GCT TCC ATT TGC ACA GCA 423

GAA GGA GCG AAC AAA TTC ATG GAA GTT GCT ATC TAT CAA GAT TAT CAT 471

GGC TTG CCA GAG TTC AGA AAT GCT GTA GCA AGG TTC ATG GAG AAG GTG 519

AGA GGT GAC AGA GTC AAG TTC GAT CCC AAC CGC ATT GTG ATG AGT GGT 567

GGG GCA ACC GGA GCT CAT GAA ACT CTG GCC TTC TGT TTA GCT GAC CCT 615

GAA GAT GCG TTT TTG GTA CCC ACA CCA TAT TAT CCA GGA TTT GAT CGG 663

GAT TTG AGG TGG CGA ACA GGG ATG CAA CTT CTT CCA ATT GTT TGT CGC 711

AGC TCC AAT GAT TTT AAG GTC ACT AAA GAA TCC ATG GAA GCT GCT TAT 759

CAG AAA GCT CAA GAA GCC AAC ATC AGA GTA AAG GGG TTC CTC TTA AAT 807

AAT CCA TCA AAT CCA TTG GGA ACT GTT CTT GAC AGG GAA ACT TTG ATT 855

GAT ATA GTC ACA TTC ATC AAT GAC AAA AAT ATC CAC TTG ATT TGT GAT 903

GAG ATA TAT TCT GCC ACC GTC TTC AGC CAG CCC GAA TTC ATC AGC ATC 951

TCT GAA ATA ATT GAG CAT GAT GTT CAA TGC AAC CGT GAT CTC ATA CAT 999

CTT GTG TAT AGC CTG TCC AAG GAC TTG GGC TTC CCT GGA TTC AGA GTT 1047

GGC ATT TTG TAT TCA TAT AAT GAC GCT GTT GTC AGC TGT GCT AGA AAA 1095

ATG TCG AGT TTC GGC CTT GTT TCA ACA CAA ACT CAG CAT CTG ATT GCA 1143

TCA ATG TTA TCG GAC GAA GCA TTT ATG GAC AAA ATC ATT TCC ACG AGC 1191

TCA GAG AGA TTA GCT GCA AGG CAT GGT CTT TTC ACA AGA GGA CTT GCT 1239

CAA GTA GGC ATT GGC ACC TTA AAA AGC AGT GCG GGC CTT TAT TTC TGG 1287

ATG GAC TTA AGG AGA CTC CTC AGG GAG TCC ACA TTT GAG GCA GAA ATG 1335

GAA CTT TGG AGG ATC ATA ATA CAT GAA GTC AAG CTC AAT GTT TCA CCA 1383

GGC TTA TCT TTC CAT TGC TCA GAA CCA GGA TGG TTC AGA GTT TGC TTT 1431

GCC AAC ATG GAC GAC GAA AGT GTG AGA GTT GCT CTC AGA AGA ATC CAC 1479

AAA TTT GTG CTT GTT CAG GGC AAG GCA ACA GAG CCA ACA ACT CCA AAG 1527

AGT CGC TGC GGA AGC AGC AAA CTT CAA CTC AGC TTA TCT TTC CGC AGA 1575

```
TTG GAC GAA AGG GTG ATG GGA TCG CAT ATG ATG TCC CCT CAC TCC CCG        1623

ATG GCT TCA CCT TTG GTT CGG GCT ACA TAAATCATTT CTTGATCAGA TCATATAGCA   1680

AAGATTCCTG AGTAAATACT CGAAACCCTT TCTGGATAAC TGAAAAGAGA GTTGTTGATT     1740
CTTTGCTGTA TCATACAAAC ACGTTACAGG CATTTTTTGG CCATCTGATG CGTGCAAATT     1800
GCATCAAATG CTTTTATTAT TGTCATATTC ATTTGTGTAC CTTGGTTTTC CTTGCCCTTC     1860
AGTCCTCCTT GTTTTTTGTT TCTTTGTTAT TATTTTCTTC CAGTTGATCA GTTAAACGAA     1920
GGAAGCTCAA TTGTTTCAAG CTATTAGTAA CAGATCATTT TGTAATAGCA ATAGTTTCAG     1980
GATTCTGAAA TGAAAGTTTA TCATTTTTCC ATCATTTTAA AAAAAAAAAA AAAAAAAAAA     2040
```

Note: The coding portion of this sequence is shown by grouping the bases as codons.

Fig. 3: DEDUCED PROTEIN SEQUENCE OF THE COFFEE FRUIT-EXPRESSED ACC OXIDASE cDNA (SEQ. ID. NO. 12)

```
                                                    Met Ala Thr Phe
                                                     1
5
     Pro Leu Ile Asp Met Glu Lys Leu Asp Gly Glu Glu Arg Ala Ala Thr
      5                  10                  15                  20

     Met Gly Val Ile Lys Asp Ala Cys Glu Ser Trp Gly Phe Phe Glu Val
10                  25                  30                  35

     Leu Asn His Gly Ile Ser Asn Glu Leu Met Asp Thr Val Glu Arg Leu
                 40                  45                  50

15   Thr Lys Glu His Tyr Lys Lys Cys Met Glu Leu Lys Phe Lys Glu Met
            55                  60                  65

     Val Glu Ser Lys Glu Leu Glu Ala Val Gln Thr Glu Ile Asn Asp Leu
          70                  75                  80
20
     Asp Trp Glu Ser Thr Phe Phe Leu Arg His Leu Pro Val Ser Asn Ile
      85                  90                  95                  100

     Ser Glu Val Pro Asp Leu Asp Asp Glu Tyr Arg Lys Val Met Lys Glu
25                  105                 110                 115
```

Phe Ala Leu Gln Leu Glu Lys Leu Ala Glu Leu Leu Leu Asp Leu Leu
120 125 130

Cys Glu Asn Leu Gly Leu Glu Lys Gly Tyr Leu Lys Lys Ala Phe Tyr
135 140 145

Gly Thr Lys Gly Pro Thr Phe Gly Thr Lys Val Ser Asn Tyr Pro Pro
150 155 160

Cys Pro Arg Pro Glu Leu Ile Lys Gly Leu Arg Ala His Thr Asp Ala
165 170 175 180

Gly Gly Ile Ile Leu Leu Phe Gln Asp Asp Lys Val Ser Gly Leu Gln
185 190 195

Leu Leu Lys Asp Gly Glu Trp Val Asp Val Pro Pro Met Arg His Ser
200 205 210

Ile Val Ile Asn Ile Gly Asp Gln Leu Glu Val Ile Thr Asn Gly Lys
215 220 225

Tyr Lys Ser Val Met His Arg Val Ile Ala Gln Pro Asp Gly Asn Arg
230 235 240

Met Ser Leu Ala Ser Phe Tyr Asn Pro Gly Ser Asp Ala Val Ile Tyr
245 250 255 260

Pro Ala Pro Ala Leu Val Glu Lys Glu Ala Glu Asp Lys Gln Ile Tyr
265 270 275

Pro Lys Phe Val Phe Glu Asp Tyr Met Lys Leu Tyr Ala Gly Leu Lys
280 285 290

Phe Gln Ala Lys Glu Pro Arg Phe Glu Ala Met Lys Ala Val Glu Ser
295 300 305

Thr Val Asn Leu Gly Pro Ile Ala Thr Val
310 315

Fig 4: DNA SEQUENCE OF THE COFFEE FRUIT-EXPRESSED ACC OXIDASE CDNA. (SEQ. ID. NO. 13)

TGTAAACGAA GCATAAGCAC AAGCAAACAC AAACTAGAAA GAGAG ATG GCT ACA TTC 57

CCC CTA ATC GAC ATG GAG AAG CTT GAC GGT GAA GAG AGG GCT GCC ACT 105

ATG GGA GTC ATA AAA GAT GCT TGT GAA AGC TGG GGC TTC TTT GAG GTG 153

TTG AAT CAT GGG ATA TCT AAT GAG CTC ATG GAC ACA GTG GAG AGG CTA 201

ACA AAG GAG CAT TAC AAG AAA TGT ATG GAA CTA AAG TTC AAG GAA ATG 249

GTG GAG AGC AAG GAA TTG GAA GCT GTT CAG ACT GAG ATC AAT GAT TTG 297

GAC TGG GAA AGT ACC TTC TTC TTG CGC CAT CTT CCT GTT TCC AAC ATC 345

TCA GAA GTC CCT GAT CTT GAT GAT GAA TAC AGA AAG GTT ATG AAG GAA 393

TTT GCG TTG CAA CTT GAG AAA CTA GCA GAG CTC CTG TTG GAC TTG CTA 441

TGC GAG AAC CTT GGC CTA GAG AAA GGC TAT CTG AAG AAA GCC TTC TAT 489

GGC ACC AAA GGA CCA ACC TTT GGC ACC AAA GTC AGC AAT TAC CCT CCA 537

TGC CCT CGT CCA GAA CTG ATC AAG GGC CTC CGG GCA CAC ACC GAT GCC 585

GGC GGC ATC ATC CTG CTG TTC CAG GAT GAC AAG GTC AGC GGT CTC CAG 633

CTC CTC AAG GAT GGT GAA TGG GTG GAT GTT CCG CCT ATG CGC CAC TCC 681

ATT GTA ATC AAC ATC GGC GAC CAA CTT GAG GTA ATC ACA AAT GGA AAA 729

TAC AAG AGT GTG ATG CAC CGG GTG ATA GCT CAA CCA GAT GGG AAC AGA 777

ATG TCA CTA GCA TCA TTC TAC AAT CCA GGA AGT GAT GCA GTG ATC TAT 825

CCA GCA CCG GCA TTG GTT GAG AAA GAG GCA GAG GAC AAG CAG ATA TAT 873

CCC AAG TTT GTG TTC GAG GAC TAC ATG AAG CTC TAT GCT GGC CTT AAG 921

TTC CAA GCT AAA GAG CCC AGG TTT GAA GCC ATG AAG GCC GTG GAA AGC 969

ACC GTA AAC TTG GGT CCA ATC GCA ACT GTT TGAGATAATA CACGCTTTGA 1019

TCTGCTGCTG TCTTATAATG CGCGTTTGCG TAATCATATC CTAGCATAGT ATATCTGAGA 1079

TCTGAGTCTG TATTGTGGTG TGAGTTTGGT TTAGCCCCTT GTTAATGCTT GGATTGGACT 1139

AGTTAAATGT GGAGCTGGTT TGTTAGATAA GATAGTCTTG CCAGGATCTT TGAGTAAATA 1199

TGATTCTGCG GAAGTCTGCG GTGAATGATA ACGTGTAAAG CAATCCGAAA GTTACCTTTC 1259

TGGGGCTTTG TCATATGCAA TGGAGAAGGA ATCTTCCAAA AAAAAAAAAA AAAAAAAAAA 1319

A 1320

Note: The coding portion of this sequence is shown by grouping the bases as codons.

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/US 97/14184

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/52 C12N15/53 C12N15/11 C12N15/82 C12N9/02
C12N9/88 C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 19103 A (DNA PLANT TECHN CORP) 27 June 1996 * see esp. pp.16-19, pp.23/24, ex.9,10 *	13-21, 28,29, 31,32
A	WO 91 01375 A (ICI PLC) 7 February 1991 see the whole document	1-33
A	WO 92 04456 A (US OF AMARICA REPRESENTED BY T) 19 March 1992 see the whole document	1-33
A	WO 96 07742 A (ASGROW SEED CO ;BOESHORE MAURY L (US); DENG ROSALINE Z (US); CARNE) 14 March 1996 see the whole document	1-33

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
2 December 1997	19/12/1997

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kania, T

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INTERNATIONAL SEARCH REPORT

Inter: nal Application No
PCT/US 97/14184

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropnate, of the relevant passages	Relevant to claim No.
A	WO 96 21027 A (ASGROW SEED CO ;BOESHORE MAURY L (US); DENG ROSALINE Z (US); CARNE) 11 July 1996 see the whole document	1-33

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INTERNATIONAL SEARCH REPORT

...formation on patent family members

Interr ...al Application No
PCT/US 97/14184

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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WO 9101375 A	07-02-91	AU 627063 B	13-08-92
		AU 6042390 A	22-02-91
		EP 0482053 A	29-04-92
		JP 4506602 T	19-11-92
		US 5530190 A	25-06-96
		US 5365015 A	15-11-94
WO 9204456 A	19-03-92	AU 657276 B	09-03-95
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WO 9621027 A	11-07-96	AU 2769395 A	24-07-96
		EP 0801681 A	22-10-97

INTERNATIONAL SEARCH REPORT

Inter: nal Application No
PCT/US 97/14184

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 21027 A (ASGROW SEED CO ;BOESHORE MAURY L (US); DENG ROSALINE Z (US); CARNE) 11 July 1996 see the whole document	1-33

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		CA 2198708 A	14-03-96
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WO 9621027 A	11-07-96	AU 2769395 A	24-07-96
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